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METABOLIC SIGNIFICANCE IN NUCLEIC ACID METABOLISM AND PROTEIN SYNTHESIS OF DIETARY AMP REQUIREMENT IN *ARTEMIA SALINA* (L.)

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The absolute and specific requirement of *Artemia* for a dietary purine nucleotide has been demonstrated. AMP deficiency reduces growth rate and survival percentage, induces a supernumerary gonopode morphogenesis and reduces abdominal length. The purine requirement can be satisfied by IMP, AMP or GMP; adenine, guanine and adenosine are metabolized but fulfill the requirement less effectively (Hernandorena, 1972b). The need for an energetic nutrient plus AMP/albumin ratio has been shown. With increasing salinity of the medium, growth rate is reduced, the abdomen grows relatively longer and the energetic nutrient plus AMP/albumin ratio is altered. The quantitative nature of AMP deficiency inducing the morphogenetic action depends on albumin concentration and salinity. Since oxidative metabolism and ATP production are required for amino acid incorporation into tissue proteins we suggested that AMP morphogenetic action should be looked for at the protein synthesis level (Hernandorena, 1974a).

The use of specific inhibitors seems to offer a specially useful approach to study the possible biochemical alterations underlying the AMP morphogenetic action. We have already effectively employed aminopterin and 5-fluorodeoxyuridine (5-FUDR) to elucidate the significance of *Artemia* folic acid requirement (Hernandorena, 1970, 1972a).

The essentiality of axenic conditions in these studies has to be underlined. The lack of activity of a number of analogues on *Oniscus asellus* growth suggests the existence of a high level of metabolite synthesis in the gut of this crustacean (Beerstecher, Cornyn and Vokmann, 1954). Fautrez-Firlefyn and Fautrez (1970) reported the remarkable lack of toxicity of hydroxyurea in *Artemia*, since viability is not affected by 48-hour immersion in M/100 and M/50 solutions.

In *Artemia*, feeding levels show a profound effect on growth both as it influences growth rate and final size attained (Mason, 1963). According to Dagg (1969), "*Artemia* protein concentration is highest during exponential growth indicating that almost all growth is due to new protein synthesis . . . the growth rates associated to a given RNA concentration range tremendously. The poor relationship between RNA/protein concentration and growth measured by protein increase is unexplainable. . . . The RNA-growth relationship is probably most valid under steady growth conditions". These are not the conditions used by the author.

In this paper we report the effects of various inhibitors affecting nucleic acid metabolism (Fig. 1) and protein synthesis. Our next paper will be concerned with the metabolic significance of dietary AMP in energy production in relation to temperature and salinity.

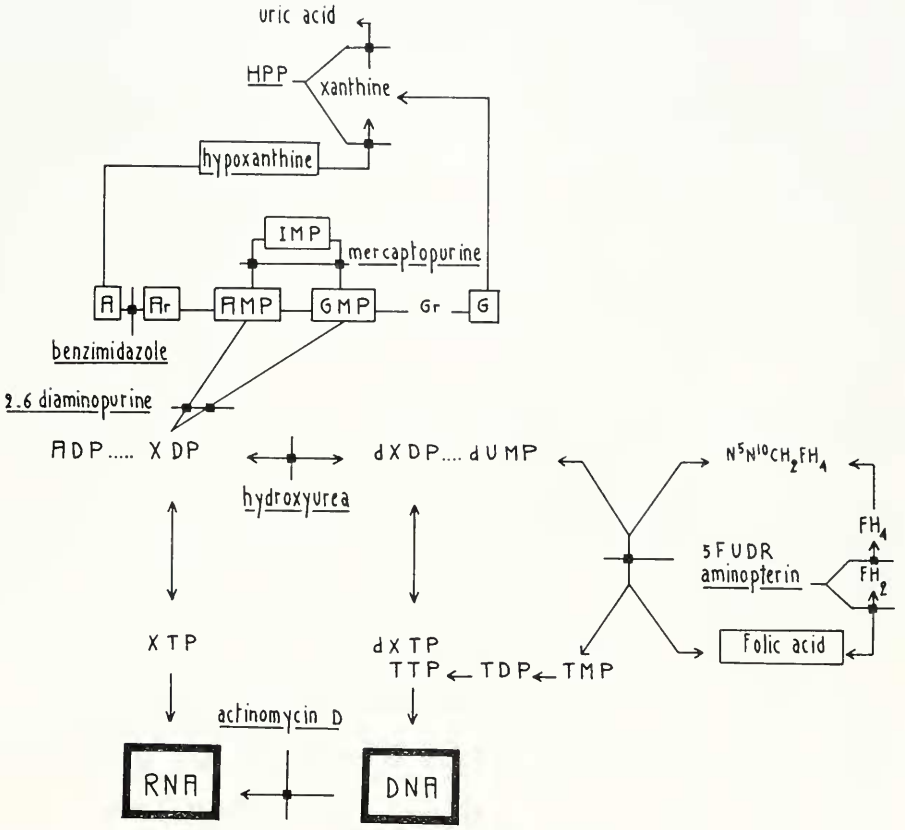

FIGURE 1. Metabolic reactions of nucleic acid, as blocked by the inhibitors administrated, are shown; metabolites administered are indicated by boxed compounds; inhibitors are underlined; and points of inhibition are indicated by a solid square on the crossing lines.

MATERIALS AND METHODS

The method developed by Provasoli and d'Agostino (1969) for the Utah strain axenic cultivation has been used and described previously (Hernandorena, 1970, 1972a, b, c). The inhibitors used are from Nutritional Biochemical Co.; HPP was graciously supplied by Burroughs Wellcome and Co.

RESULTS

Nucleic acid metabolism

Purine catabolism leads to xanthine dehydrogenase substrates hypoxanthine and xanthine. This enzyme is specifically inhibited by 4-hydroxy-pyrazolo-(3-4 d)-pyrimidine (HPP) and this inhibitor action has already been studied in *Artemia* (Hernandorena, 1972c). We intend to study HPP action on larvae for which purine requirement has been fulfilled by different purine derivatives.

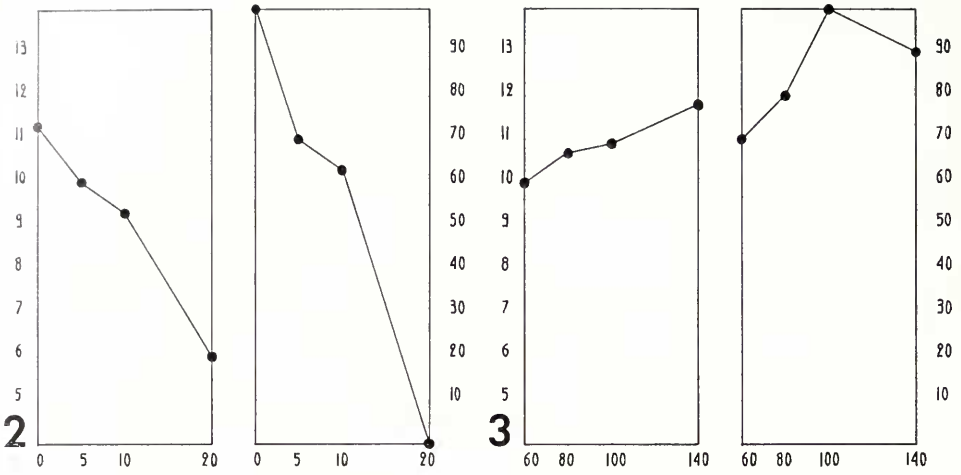

FIGURE 2. Effect of benzimidazole; both abscissae represent benzimidazole in mg%. In all figures, growth is represented in the left-hand, and survival in the right-hand graph, the left-hand ordinate being the growth index for the 14th day of development, and the right-hand ordinate being the survival percentage for index 10 (end of the larval life), see Hernandorena, 1972a.

FIGURE 3. Benzimidazole-AMP antagonism; both abscissae AMP in mg%; benzimidazole constant at 5 mg%; ordinates growth and survival as in Figure 2.

Benzimidazole reduces growth index and survival percentage (Fig. 2) but does not induce a supernumerary gonopode morphogenesis. The detrimental effect of benzimidazole is relieved by AMP (Fig. 3).

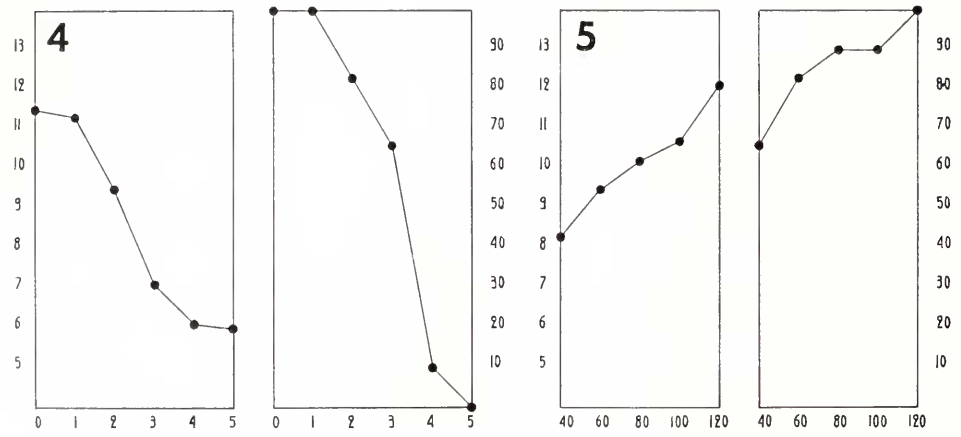

FIGURE 4. Effect of 2-6 diaminopurine; both abscissae 2-6 diaminopurine in mg%; ordinates growth and survival as in Figure 2.

FIGURE 5. 2-6 diaminopurine-AMP antagonism; both abscissae AMP in mg%; 2-6 diaminopurine constant at 2 mg%; ordinates growth and survival as in Figure 2.

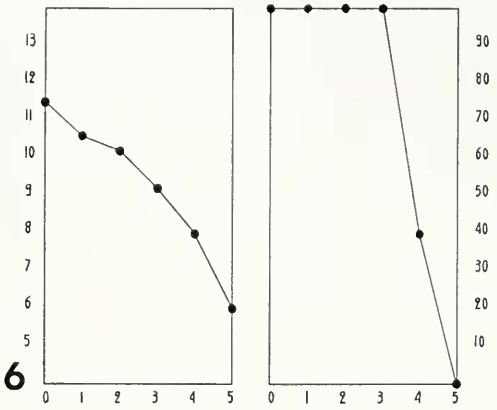

FIGURE 6. Effect of mercaptopurine; both abscissae mercaptopurine in mg%; no AMP present, IMP at 60 mg%; ordinates growth and survival as in Figure 2.

The inhibitory effect of 2–6 diaminopurine administration (Fig. 4) is relieved by AMP (Fig. 5). Morphogenesis is normal.

The 6-mercaptopurine action has been studied on larvae reared in a medium containing 60 mg% IMP instead of AMP. Figure 6 shows the detrimental effect of mercaptopurine: it reduces growth index and survival percentage but does not induce a supernumerary gonopode morphogenesis.

Figure 7 shows the inhibitory effect of hydroxyurea administration; this effect is not relieved by AMP (Fig. 8).

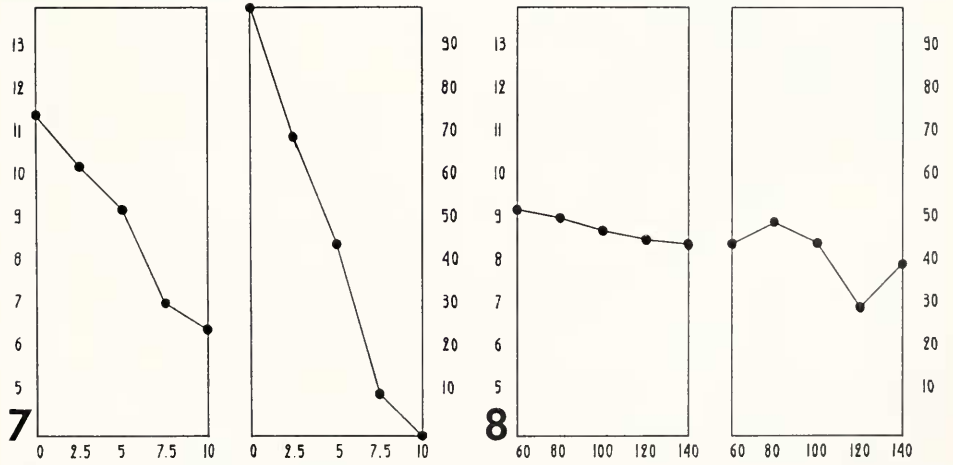

FIGURE 7. Effect of hydroxyurea; both abscissae hydroxyurea in mg%; ordinates growth and survival as in Figure 2.

FIGURE 8. Hydroxyurea-AMP antagonism; both abscissae AMP in mg%; hydroxyurea constant at 5 mg%; ordinates growth and survival as in Figure 2.

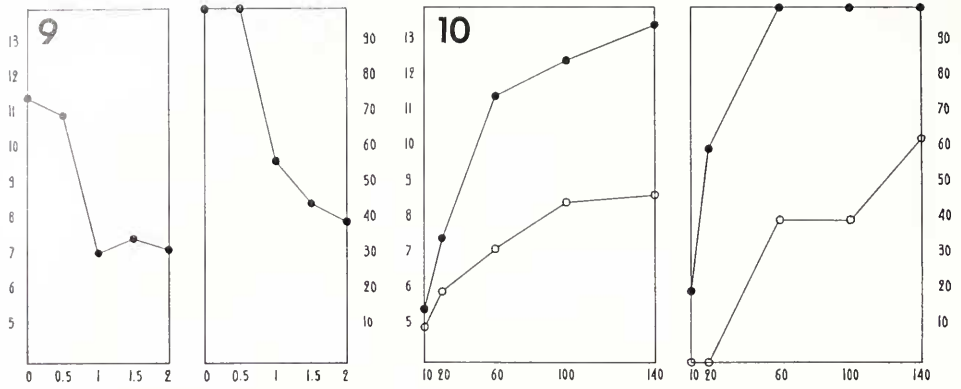

FIGURE 9. Effect of actinomycin D; both abscissae actinomycin in μg%; ordinates growth and survival as in Figure 2.

FIGURE 10. Effect of AMP concentration; both abscissae AMP in mg%; closed circles, no addition; open circles, actinomycin D 2 μg%; ordinates growth and survival as in Figure 2.

Actinomycin D reduces growth index and survival percentage (Fig. 9) and this action is dependent on AMP concentration (Fig. 10). This antibiotic does not induce a supernumerary gonopode morphogenesis. The result has been checked by rearing larvae in an actinomycin D-containing medium during different periods of the larval life. Morphogenesis is normal whatever the time spent in the presence of the antibiotic.

HPP action depends on AMP concentration (Fig. 11) and on adenine concentration (Fig. 12). Minimal action occurs at 100 mg% AMP and at a 10

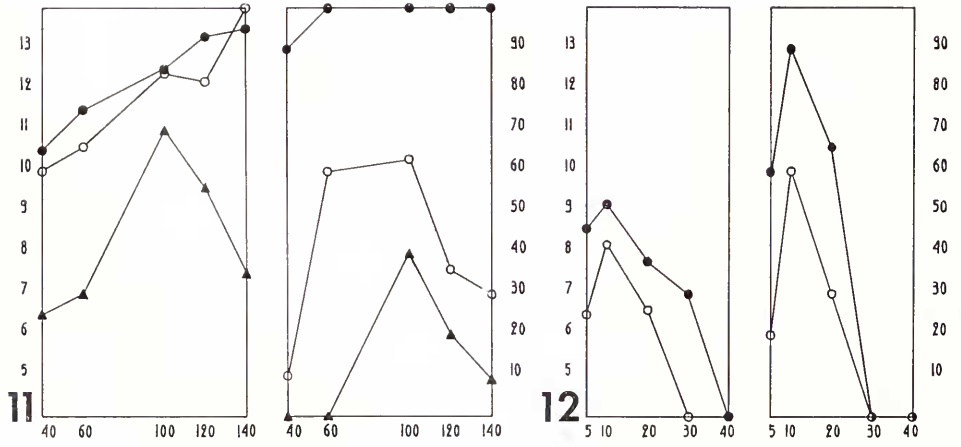

FIGURE 11. Effect of AMP concentration; both abscissae AMP in mg%; closed circles, no addition; open circles, HPP at 40 mg%; closed triangles, HPP at 80 mg%; ordinates growth and survival as in Figure 2.

FIGURE 12. Effect of adenine; both abscissae adenine in mg%; closed circles, no addition; open circles, HPP at 20 mg%; ordinates growth and survival as in Figure 2.

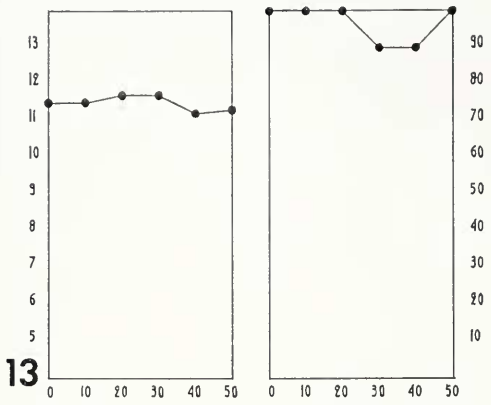

FIGURE 13. Effect of hypoxanthine; both abscissae hypoxantine in mg%; ordinates growth and survival as in Figure 2.

mg% adenine concentration. With increasing AMP or adenine concentration HPP action increases.

Hypoxanthine administration up to a 50 mg% level has no effect on growth rate and survival percentage (Fig. 13).

Protein synthesis

Protein synthesis has been studied by the use of the antibiotic puromycin and the amino acid analogue D.L. parafluorophenylalanine under different experimental conditions.

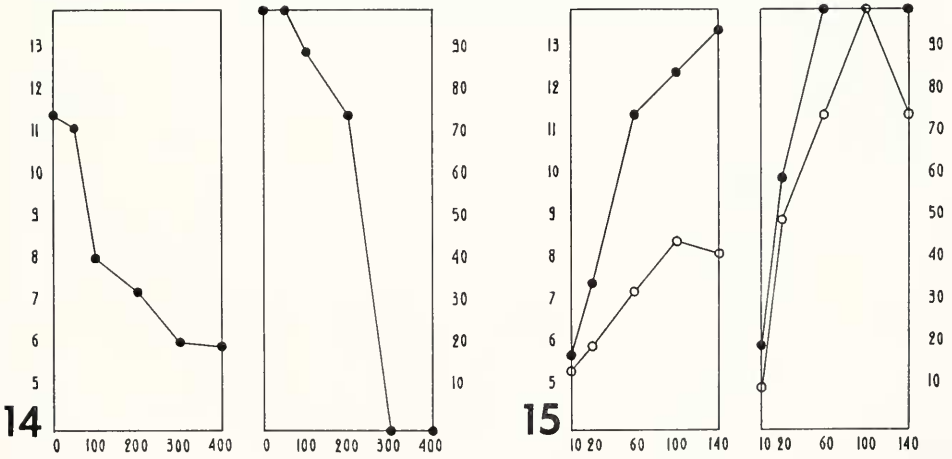

FIGURE 14. Effect of puromycin; both abscissae puromycin in μg%; ordinates growth and survival as in Figure 2.

FIGURE 15. Effect of AMP concentration; both abscissae AMP in mg%; albumin constant at 20 mg%; closed circles, no addition; open circles, puromycin at 200 μg%; ordinates growth and survival as in Figure 2.

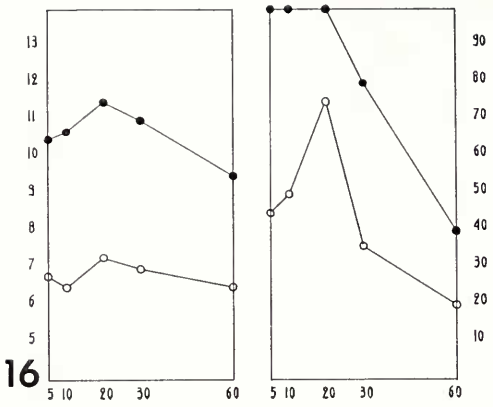

FIGURE 16. Effect of albumin concentration; both abscissae albumin in mg%; AMP constant at 60 mg%; closed circles, no addition; open circles, puromycin at 200 μg%; ordinates growth and survival as in Figure 2.

Puromycin adminstration reduces growth index and survival percentage (Fig. 14).

With albumin constant at 20 mg%, puromycin action decreases with increasing AMP concentration up to a 100 mg% level (Fig. 15).

With AMP constant at 60 mg%, puromycin action depends on albumin concentration, the minimal effect corresponding to a 20 mg% level (Fig. 16).

With increasing salinity, albumin constant at 20 mg% and AMP constant at 60 mg%, puromycin detrimental effect increases (Fig. 17).

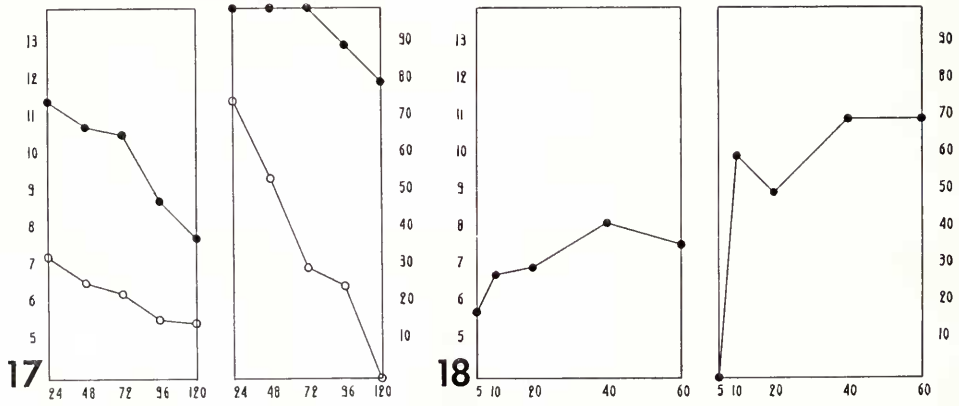

FIGURE 17. Effect of salinity; both abscissae NaCl in g‰; albumin constant at 20 mg%; AMP constant at 60 mg%; closed circles, no addition; open circles, puromycin at 200 μg%; ordinates growth and survival as in Figure 2.

FIGURE 18. Effect of albumin concentration; both abscissae albumin in mg%, salinity at 120‰; closed circles, puromycin at 50 μg%; ordinates growth and survival as in Figure 2.

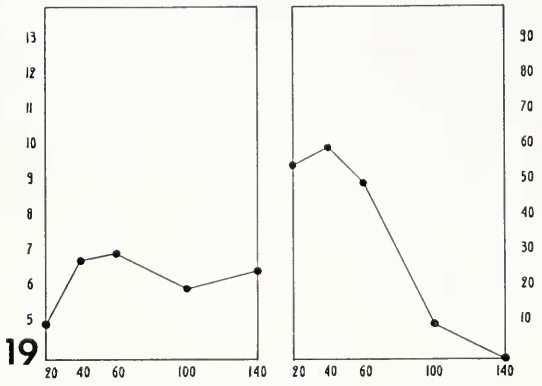

FIGURE 19. Effect of AMP concentration; both abscissae AMP in mg%; salinity at 120‰; closed circles, puromycin at 50 μg%; ordinates growth and survival as in Figure 2.

We have demonstrated that with increasing salinity, the albumin requirement increases and that the growth index increases with AMP concentration, provided enough albumin is supplied (Hernandorena, 1974a).

With salinity constant at 120‰, puromycin action depends on albumin concentration (Fig. 18) and AMP concentration (Fig. 19), minimal effect corresponding to a 40 mg% albumin level and a 40 mg% AMP concentration.

These results are confirmed by D.L. parafluorophenylalanine administration.

This analogue reduces growth index and survival percentage (Fig. 20).

At 24‰ salinity, minimal action corresponds to a 100 mg% AMP concentration (Fig. 21) and to a 20 mg% albumin concentration (Fig. 22).

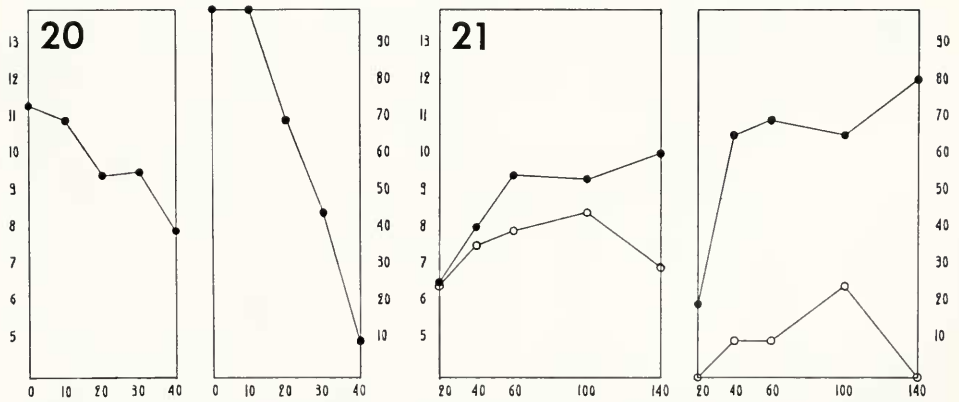

FIGURE 20. Effect of D.L. parafluorophenylalanine; both abscissae parafluorophenylalanine in mg%; ordinates growth and survival as in Figure 2.

FIGURE 21. Effect of AMP concentration; both abscissae AMP in mg%; albumin constant at 20 mg%; closed circles, parafluorophenylalanine 20 mg%; open circles, parafluorophenylalanine 40 mg%; ordinates growth and survival as in Figure 2.

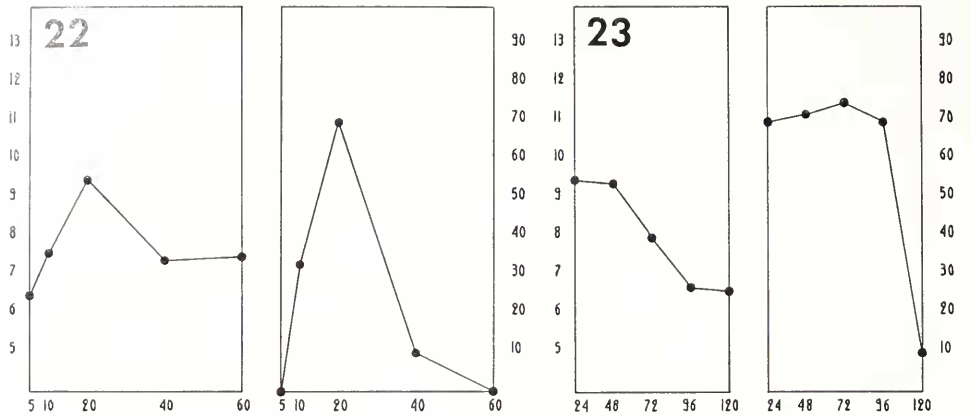

FIGURE 22. Effect of albumin concentration; both abscissae albumin in mg%; AMP constant at 60 mg%; closed circles parafluorophenylalanine 20 mg%; ordinates growth and survival as in Figure 2.

FIGURE 23. Effect of salinity; both abscissae NaCl in g‰; albumin constant at 20 mg%, AMP constant at 60 mg%; closed circles, parafluorophenylalanine 20 mg‰; ordinates growth and survival as in Figure 2.

With increasing salinity, parafluorophenylalanine detrimental effect increases (Fig. 23). At 120‰ salinity, minimal action corresponds to a 40 mg% AMP concentration (Fig. 24), and to a 40 mg% albumin concentration (Fig. 25), thus confirming the reduction in AMP requirement and increase in albumin requirement with increasing salinity.

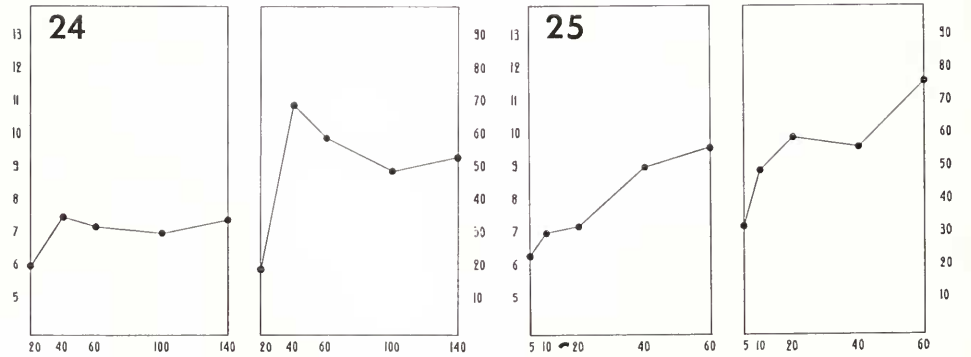

FIGURE 24. Effect of AMP concentration; both abscissae AMP in mg%; salinity at 120‰; closed circles, parafluorophenylalanine at 5 mg%; ordinates growth and survival as in Figure 2.

FIGURE 25. Effect of albumin concentration; both abscissae albumin in mg%; salinity at 120‰; closed circles, parafluorophenylalanine at 5 mg%; ordinates growth and survival as in Figure 2.

Discussion

Since the specificity and essentiality of a purine nucleotide requirement has already been demonstrated by omission experiments (Hernandorena, 1972b), the growth inhibition observed after administration of the different inhibitors, does not bring any additional data. No morphogenetic effect is induced whatever the reaction blocked, and this negative result underlines the limits of the antagonists method.

The morphogenetic effects observed after DNA synthesis inhibition resulting from aminopterin or 5-FUDR administration have been described (Hernandorena, 1970, 1972a). We observed a teratogenic action with numerous appendicular malformations which are different from the supernumerary gonopodes induced by AMP deficiency. We can conclude that the morphogenetic action caused by AMP deficiency does not result from inhibition of DNA synthesis. This is confirmed by hydroxyurea administration, since hydroxyurea induced growth inhibition is not relieved by AMP.

Actinomycin D action depends on AMP concentration and this antibiotic does not induce any morphogenetic action.

We have demonstrated that a critical period exists for the induction of supernumerary gonopods. Animals reared on an AMP-deficient medium until growth index 7 and transferred at this stage to the basal medium show supernumerary gonopods like animals reared on an AMP-deficient medium until maturity (Hernandorena, 1974b). Actinomycin D administration restricted to the critical period does not induce supernumerary gonopods. This negative result underlines the problem of interpreting antibiotics action.

It is not clear how nucleotides would be superior to the bases or nucleosides since the former group of compounds are not effectively transported across cell membranes and probably must be catabolized to the nucleosides or bases prior to entering the cell (Kelley, 1972). We suggested that dietary bases are catabolized too rapidly (Hernandorena, 1972b). HPP action or xanthine dehydrogenase activity is dependent on the qualitative and quantitative nature of dietary purine derivatives. Hypoxanthine has no effect and this metabolite would not be metabolized (Stirpe and Della Corte, 1965). When adenine is supplied to meet the purine requirement, HPP action increases. This result could be interpreted as suggested by a rapid catabolism of dietary bases.

It is interesting to note that in different insects, xanthine dehydrogenase activity has been shown to vary with diet, especially with the protein composition of the diet (Ito and Mukaiyama, 1964; Villela, Calcagnotto, Piedras Lopes, and Rios Magalhaes, 1970) and that the dietary RNA requirement depends on the protein concentration of the diet (Geer, 1963).

In *Artemia* the quantitative nature of AMP requirement depends on the albumin concentration of the diet (Hernandorena, 1974a).

With albumin constant at 20 mg%, and provided enough energetic nutrients are supplied, growth rate increases with the AMP concentration up to a 140 mg% level and decreases thereafter. The major increase takes place between 20 and 60 mg% AMP (Hernandorena, 1974a). The abdomen grows relatively longer with increasing AMP concentration up to a 200 mg% level. With AMP concen-

TABLE I

Growth rate and abdominal length as percentage of total length (1 % L) in relation to AMP concentration and salinity.

Albumin constant at 20 mg%	Salinity						
Starch constant at 100 mg%	24‰						120‰
	AMP mg%						AMP mg%
	20	60	100	140	180	200	60
Growth index 14th day	7.5	11.5	12.5	13.5	12.3	11.7	7.8
1 % L	39.7	45.2	47.6	48	50	54	49.3

tration ranging from 140 mg% to 200 mg% growth rate decreases but abdominal length increases.

With AMP constant at 60 mg% and albumin constant at 20 mg% growth rate decreases and abdominal length increases with increasing salinity. These results are summarized in Table I. Part of them will be published separately. The abdominal length as percentage of total length (1% L) is reported and measured using the methods described by Gilchrist (1956).

These results show that AMP deficiency reduces growth index and abdominal length while salinity reduces growth index and increases abdominal length. So it seems that growth rate and abdominal length depend on two different metabolic systems.

At 24‰ salinity, maximal protein synthesis estimated by minimal puromycin and parafluorophenylalanine action is achieved with an AMP:albumin ratio standing somewhere at 100:20. This ratio does not correspond to maximal growth rate which is achieved with a 140:20 ratio, nor to maximal abdominal length which is achieved with a 200:20 ratio.

With increasing salinity, puromycin and parafluorophenylalanine detrimental effect increases. This result suggests that protein synthesis decreases with increasing salinity. We have demonstrated that protein synthesis depends on albumin concentration but albumin requirement increases with increasing salinity. With albumin constant at 20 mg% protein synthesis would decrease with increasing salinity.

Survival studies of newly hatched nauplii, incubated during 24 hours at various salinities in the presence of puromycin, chloramphenicol and cycloheximide, demonstrated that protein synthesis inhibitors are more effective at high salt concentration. The results are not due to changes in permeability to the inhibitors with varying salinity since there is no significant difference in the uptake of ^{3}H-puromycin by nauplii incubated at different salinities (Ewing, Peterson and Conte, 1972). In our experimental conditions, animals are feeding and the results obtained could be due to a different drinking rate resulting from the mechanisms of osmotic regulation (Hernandorena, 1974a) although Croghan (1958) stated that swallowing is continuous in all active animals whatever the salinity of the medium.

With salinity constant at 120‰, that is to say with a constant drinking rate, maximal protein synthesis is achieved with an AMP:albumin ratio standing somewhere at 40:20. So it can be concluded that for a given albumin concentration (20 mg%), salinity reduces the AMP requirement for maximal protein synthesis.

In *Bombyx mori*, larval growth depends on at least two metabolic systems; one associated with the length of early developmental period which is specially sensitive to carbohydrate metabolism, and the other responsible for an extra larval molting which is probably related to protein metabolism (Kato and Sumimoto, 1968). With increasing salinity, *Artemia* starch requirement decreases (Hernandorena, 1974a).

Before drawing any interpretation, conversion of these informations to energy flow must be introduced. The ratio between AMP concentration and ATP production should be considered. So information must be gained regarding the nature of substrates oxidized.

As pointed by House (1966), Sang (1959) stated that balance between nutrients deserves much more investigation because here one delves most closely into examination of metabolic processes. The influence of metabolic adaptation to salinity and temperature on the energetic nutrient plus AMP/albumin ratio might bring more data than inhibitor studies to explain how quantitative differences in AMP concentration can be translated at the morphological level.

Summary

The metabolic reactions of the nucleic acids blocked by benzimidazole, 2–6 diaminopurine, 6 mercaptopurine, hydroxyurea, actinomycin D and HPP are essential for *Artemia* growth and survival.

The morphogenetic action of AMP deficiency is not induced by the administration of any of these antagonists. Xanthine dehydrogenase activity has been shown to vary with the quantitative and qualitative nature of the dietary purine derivatives.

At 24‰ salinity maximal protein synthesis estimated by minimal puromycin and parafluorophenylalanine action depends on the AMP and albumin concentrations of the diet and corresponds to a 100:20 ratio.

At 120‰ salinity the optimal AMP:albumin ratio stands at 40:20. Salinity reduces the AMP requirement for maximal protein synthesis. The data presented suggest that AMP concentration controls growth rate and abdominal length through different metabolic systems.

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